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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Radiation therapy is both a common and effective strategy for the treatment of localized prostate cancer. However, a proportion of locally advanced cancers develop radiation resistance and recur after therapy, therefore the development of radiation sensitizing compounds is essential for treatment of these tumors. DAB2IP (DOC-2/DAB2 interactive protein) which is a novel member of the Ras GTPase-activating protein family and a regulator of PI3K/Akt activity, is often downregulated in aggressive prostate cancer (PCa). A novel DNA-PKcs inhibitor NU7441 can significantly enhance the effect of radiation in DAB2IP-deficient PCa cells. This enhanced radiation sensitivity after NU7441 treatment is primarily due to delayed DNA double-strand break (DSB) repair. More importantly, we reported that DAB2IP-deficient PCa cells show dramatic induction of autophagy after treatment with radiation and NU7441. Immunoblotting analysis showed that the autophagy-associated proteins such as LC3B and Beclin1 deregulated in DAB2IP proficient PCa cells. We observed decreased phosphorylation of S6K and mTOR in DAB2IP-overexpressed cells. Taken together, our study clearly shows that NU7441 is a potent radiosensitizer in aggressive PCa cells. More importantly, our study indicates that DAB2IP may act as an important factor in PCa cell death after combined treatment with NU7441 and radiation.

15. SUBJECT TERMS
Radiation therapy, Prostate cancer,Radio-sensitization, DNA-Double strand break, cell cycle

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Introduction

Prostate cancer (PCa) is the second leading cause of cancer death (~30,000/year) in men in the USA. Surgery and radiotherapy are the most effective therapies to treat PCa patients. However, both these forms of treatment, show significant tumor recurrence with locally aggressive disease, metastasis and the morbidity in patients. Several biological disorders are thought to underlie the cause of prostate cancer. One such factor is a tumor suppressor gene DAB2IP which encodes a member of the Ras-GAP protein family. Genome wide Single Nucleotide Polymorphism (SNP) association studies in a large number of patients indicated that DAB2IP is linked with the risk of aggressive prostate cancer. DAB2IP deficient PCa cells are resistant to radiation treatment. Therefore, to improve radiation killing of these aggressive PCa cells, this proposal will explore the radiosensitizing property of NU7441, a specific kinase inhibitor of DNA-PKcs. Three specific aims were proposed:

Aim 1: To test whether a DNA-PKcs inhibitor can radio-sensitize DAB2IP deficient aggressive PCa cells under normoxic and hypoxic conditions.
Aim 2: To study the role of DNA-PKcs in HIF-1α stabilization under hypoxic conditions in DAB2IP deficient prostate cancer cells.
Aim 3: To investigate the combined effect of a DNA-PK inhibitor and radiation therapy in a rodent orthotopic (OT) PCa model using image guided radiation therapy (IGRT).

We have made significant progress towards specific aim 1 and results were described in details in the following sections.

Body:

Following tasks were proposed for the Year 1.

Task 1 (Specific Aim 1.1): Months 1-12
Investigate the role of DAB2IP protein in the determination of radiation response in prostate cancer cells. We will perform in vitro experiments using the functional domains of DAB2IP that will be over-express in prostate cancer cells. Radiation response will be established by clonogenic survival assay, DNA repair kinetics, apoptosis and cell cycle arrest. Appropriate cell lines will be established prior to analyze radiation response. Ms. Gore in will be responsible for developing the cell lines under Dr. Hseih's supervision and Ms. Gore will coordinate weekly with Dr. Mathur in Saha's. Once these cell lines are established Drs. Saha and Chen's lab will perform all the radiation related experiments.

Task 2 (Specific Aim 1.2): Months 1-6
We will examine the efficacy of NU7441 as a potential radiosensitizer in the DAB2IP deficient prostate cancer cells. Both normoxic and hypoxic studies will be performed in with or without NU7441. We will mostly performed the clonogenic surviving fraction analysis under these conditions. These studies will be primarily performed in Dr. Saha's lab. Dr.Saha along with Dr. Mathur will finish these studies.

Task 3 (Specific Aim 1.3): Months 4-10
We will investigate the mechanism of radiosensitization by NU7441 in DAB2IP deficient prostate cancer cells. DNA DSB repair kinetics, apoptosis, cell cycle analysis will be studied by Dr. Lin under Dr. Chen's supervision. Dr. Mathur and Dr. Lin will coordinate closely while performing these experiments.

Task 4 (progress report and manuscript preparation) Months 10-12
Dr. Saha will prepare the annual report on the 1st year progress and prepare manuscript focusing on the role of DAB2IP in prostate cancer cell radio-sensitization. Drs. Saha, Chen and Hsieh will closely work on the preparation of the manuscript.

Key research accomplishments

Task 1:
Following table demonstrates the status of various clones of C4-2 cells. These clones were prepared using the different functional domains of DAB2IP that over-expressed in prostate cancer (PCa) cells. As mentioned in the table that so far we failed to express the WT-PR domain of DAB2IP in PCa cells because of the toxicity associated with the overexpression of WT-PR domain. We are considering the

Cell lines	Status
C4-2 Neo	DAB2IP deficient (established)
C4-2D2	Express full-length DAB2IP (established)
C4-2D2-WT-PR	Express wild-type PR domain of DAB2IP (not surviving)
C4-2D2-AAA-PR	Express AAA mutant PR domain of DAB2IP (established)
C4-2D2-WT-PER	Express wild-type PER domain of DAB2IP (in process)
C4-2D2-S604A-PER	Express S604A mutant PER domain of DAB2IP (established)

alternative strategies such as conditional expression of this domain. We also have characterized the clones to examine the radiation response by clonogenic survival assay, DNA repair kinetics, apoptosis and cell cycle arrest. The results of characterization using the 1st two clones C4-2 neo and C4-2D2 were showed in Figure 2-4 and are the part of the manuscript which will be submitted to the Journal of Neoplasia. A draft of this manuscript is attached with this progress report. The follow figure (Figure 1) showed the preliminary characterization of C4-2D2-AAA-PR and C4-2D2 S604A-PER clones. Fig 1A showed the expression level of these DAB2IP domains. Fig. 1B showed the colony forming ability of these clones where as Fig. 1C is the digital images of the colony formation of the C4-2 clones.

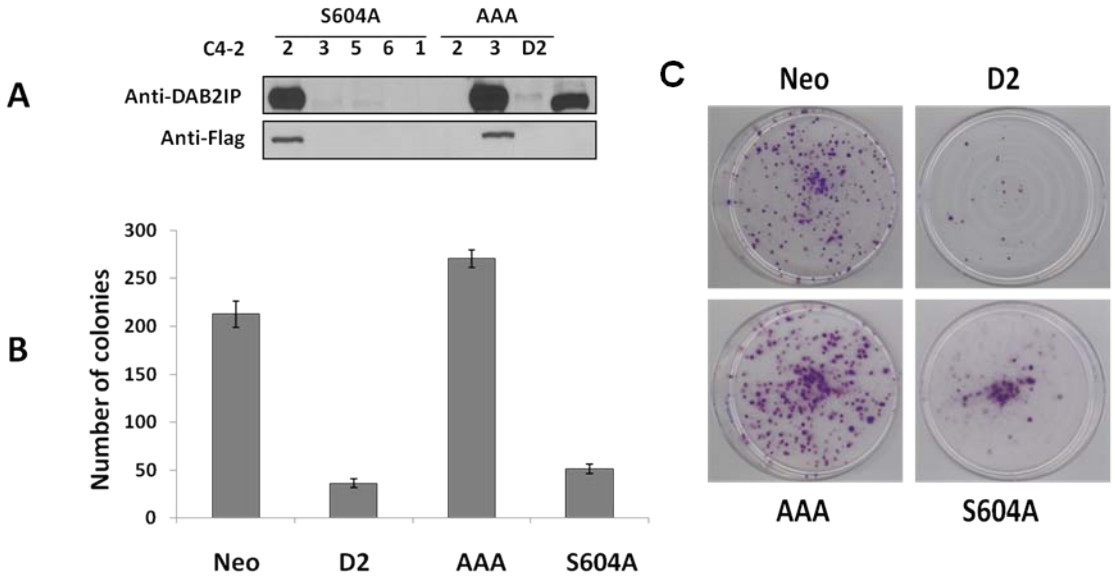

Figure 1. Characterization of C4-2 PCa cell lines expressing different functional domains of DAB2IP. (A) Immunoblot analysis of expression level of DAB2IP domains as indicated. (B). Colony forming ability of the domains determined by standard colony formation assay. (C). Digital images of the colony formation of the different clones of C4-2 cells.

Key research accomplishments

Task 2: NU7441 sensitizes DAB2IP-deficient cells to irradiation

To test the effect of NU7441 on the radiosensitivity of DAB2IP positive and negative prostate cancer cells endogenous DAB2IP expression was knocked down using shRNA in PC3 cells (PC3 KD). In addition, we stably transfected and expressed exogenous DAB2IP protein in a DAB2IP null PCa cell line C4-2 and marked as C4-2D2, whereas, the control cell line marked as C4-2 Neo. Previously we showed the immunoblot analyses to confirm DAB2IP expression in PC3 and C4-2 cells.

As shown in Fig. 2 (A, B) shRNA-mediated suppression of DAB2IP significantly increased radiation resistance in PC3 cells (Fig. 2B). In contrast, DAB2IP overexpression resulted in a significant radiation sensitizing effect in C4-2 D2 cells (Fig. 2A). Using colony formation assays, we found that NU7441 significantly sensitized DAB2IP-deficient (C4-2 neo and PC3 KD) cells to IR. Surviving fraction at 2 Gy (SF_2) for C4-2 neo and PC3 KD cells were reduced from 0.65 ±0.023 and 0.86 ±0.04 to 0.24 ±0.04 and 0.55 ±0.06 respectively. Here we confirm, as previously shown, DAB2IP expressing cells are more radiosensitive when compared to DAB2IP deficient cells. Furthermore we found that NU7441 further sensitized DAB2IP efficient cells to IR. SF_2 values of C4-2 D2 and PC3 Con vector cells were decreased from 0.31 ±0.06 and 0.58 ±0.06 to 0.14 ±0.02 and 0.02 ±0.007 respectively. The radiation sensitizing effect of NU7441 is dose dependant (Fig. 2C). These in vitro results indicate that NU7441 can radiosensitize both DAB2IP positive and negative PCa cells to radiation.

Figure 2:

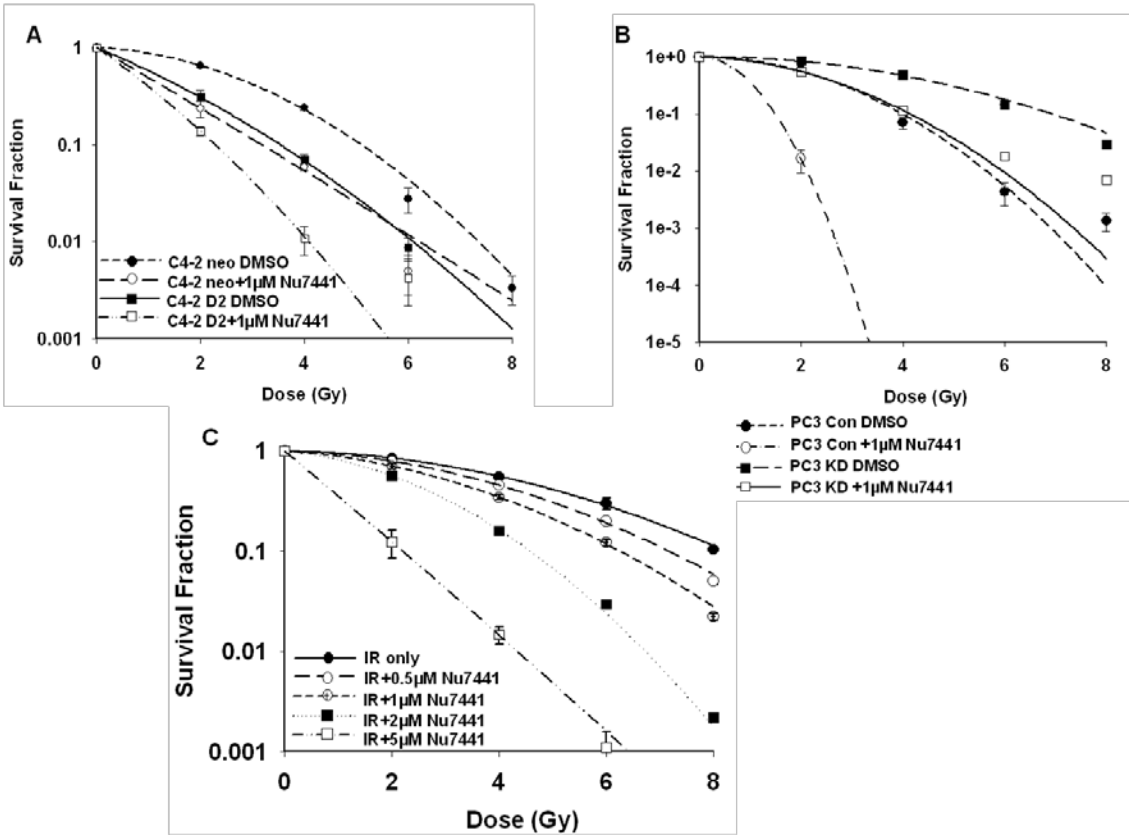

Figure 2. DNA-PKcs inhibitor NU7441 increased the radiosensitivity in both DAB2IP deficient and overexpressed prostate cancer cells to IR. (A) The survival curves of DAB2IP-overexpressed C4-2 D2 cells and control C4-2 neo cells after combination treatment with 1 μM NU7441 and irradiation at doses of 0-8 Gy. *(B)* The survival curves of DAB2IP-surrpressed PC3 KD cells and control PC3 Con cells after combination treatment with 1 μM NU7441 and irradiation at doses of 0-8 Gy. *(C)* The survival curves of PC3 KD cells after combination treatment with 0.5-5 μM NU7441 and irradiation at doses of 0-8 Gy. The data represented as the means ± standard deviations from three independent experiments.

Key research accomplishments

Task 3: NU7441 decreases DNA DSB repair in DAB2IP positive and negative PCa cells.

The extent of DNA DSBs generated and the ability of tumor cells to repair the damage largely determine the efficacy radiotherapy. Therefore, to investigate whether the radio-sensitizing effect of NU7441 on DAB2IP-deficient cells was a result of increased compromised DSBs repair, cells were subjected to immunofluorescence staining for phosphorylated histone H2AX (γH2AX) (green) and 53BP1 (red) (Fig. 3A and 3B), DNA DSBs repair kinetics were determined by counting the separate foci at each time point. In this study, C4-2 neo and C4-2 D2 cells were exposed to 2 μM Nu7441 for 1 h before 2 Gy IR treatment and the cells were collected at various times as indicated. As shown in Fig. 3 C, rapid induction of DNA damage was detected within 30 min in all of the cells. In untreated cells, DNA DSBs foci were almost abolished at 24 h after IR whereas, a significant number of DSBs remained at 24 hours in NU7441 treated cells; similar results were present in PC3 KD cells (Fig. 3B and 3D). The residual number of DSB foci per nucleus in DAB2IP overexpressed C4-2 cells was significantly higher than the control cells 30 minutes post-irradiation, however, detectable foci fell to the same level as control cells 24 hours post irradiation. This data indicates that DAB2IP overexpression in C4-2 cells promotes IR induced DNA DSBs but has no effect on repair kinetics. Taken together, these results show that NU7441 can lead to defective DNA damage repair in response to IR even in DAB2IP-deficient metastatic PCa cells.

Figure 3:

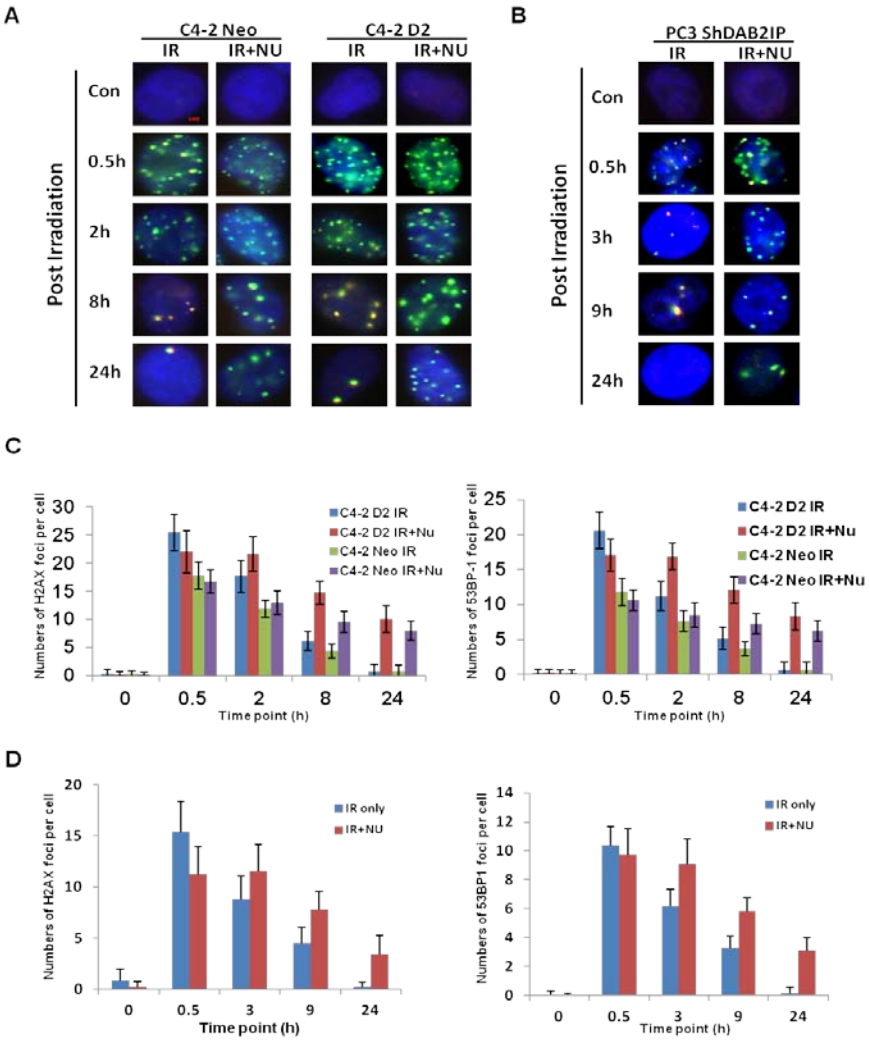

Figure 3. Decreased capacity of DNA double-strand break repair in NU7441 treated prostate cancer cell lines. *(A)* C4-2 D2 and C4-2 Con cells were treated with 2μM NU7441, 30 min later were irradiated with 2 Gy γ-ray and immunostained for 53BP1 (red) and phospho-γH2AX (green) foci at the indicated time points after irradiation. 53BP1 and phospho-γH2AX foci were counted for each time point (average, 50 nuclei). *(B)* PC3 KD cells were treated with 2μM NU7441, 30 min later were irradiated with 2 Gy γ-ray and immunostained for 53BP1 (red) and phospho-γH2AX (yellow) foci at the indicated time points after irradiation. 53BP1 and γH2AX foci were counted separated for each time point (average, 50 nuclei). *(C)* and *(D)* DNA repair kinetics between these cells was obtained by plotting the number of remaining foci against time.

Task 3: Cell Cycle Analysis

In mammalian cells, IR-induced cell cycle arrest is necessary to maintain genomic stability and is correlated to cell survival. In this study, the effect of NU7441 on cell cycle distribution was investigated by flow cytometry. As shown in Fig. 4 (A-D), treatment with 2 µM NU7441 resulted in robust G2/M arrest 24 hours after irradiation (3 Gy) in both DAB2IP-deficient or overexpressed cell lines. We further compared C4-2 neo and C4-2 D2 cells and showed the percentage of G_0/G_1 phase cells in C4-2 D2 was more than that in neo (control) line. The cell cycle results indicate that DAB2IP alone causes significant G0/G 1 arrest in C4-2 cells and is augmented after treatment with either IR or NU7441. Whereas DAB2IP deficient cells showed moderate increase in G_0/G_1 arrest after NU7441 treatment. However, both cell lines showed robust G_2/M arrest at 24 h in response to combined treatment.

Figure 4:

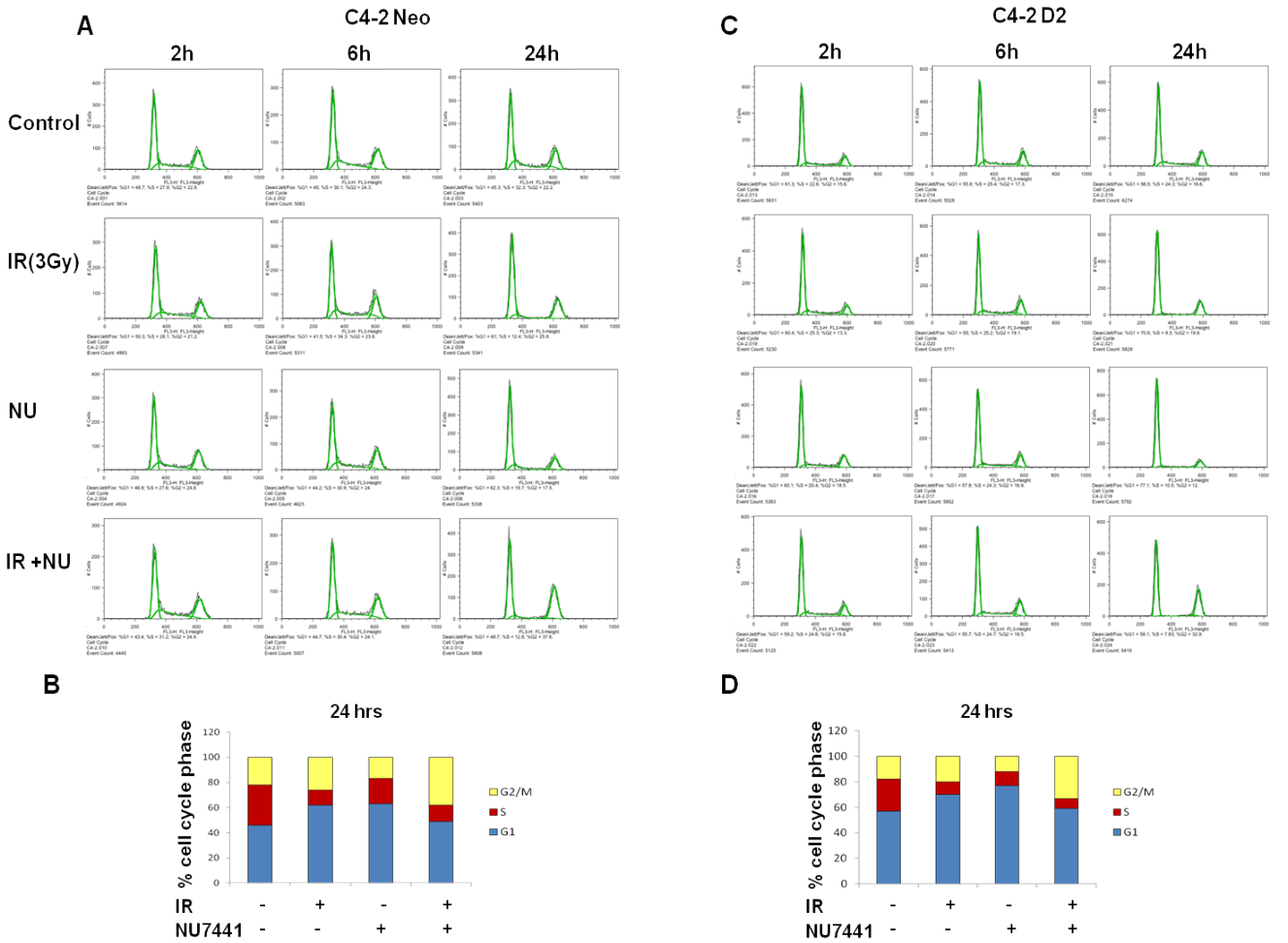

Figure 4. **Treatment with 2µM NU7441 results in a robust G2/M cell arrest 24h after 3Gy irradiation in either C4-2 Neo or C4-2 D2 cells.** *(A)* C4-2 D2 and C4-2 neo cells were treated with 2µM NU7441 or combination treated with 3 Gy and collected 0, 2, 6, and 24 h post-treatment. PI staining was applied to detect the distribution of cell cycle response to ionizing irradiation by flow cytometry. *(B)* Cell cycle distribution of cells 24h after NU7441 treatment and (or) 3 Gy irradiation. (B-D): Represents the % cell cycle phases calculated from panel A and B.

Task 3: Cellular apoptosis

We have analyzed cellular apoptosis in response to the combined treatment of IR and NU7441 in C4-2 neo and C4-2D2 prostate cancer cells to investigate the mechanism of radiosensitization. For this study we performed both Annexin V-PE staining (Fig. 5B) and TUNEL (Fig. 5C). In both experiments we noticed that combined treatment of IR and NU7441 caused significant apoptosis in either C4-2 neo (DAB2IP deficient) or C4-2D2 (DAB2IP proficient) prostate cancer cells. We also noticed that greater number of C4-2D2 cells (13.9%) is undergoing apoptosis in response to IR compare to C4-2 Neo (9.7%). Similarly, C4-2D2 cells (17.9%) are more apoptotic than C4-2 Neo (12.4%) in response to IR+NU7441 (Fig 5B). TUNEL assay also confirmed more apoptotic cell death in response to IR+NU7441 (Fig 5C). These results were further confirmed by westernblot analysis by showing PARP-1 cleavage and cleaved Caspase-3 (Fig 5A).

Figure 5:

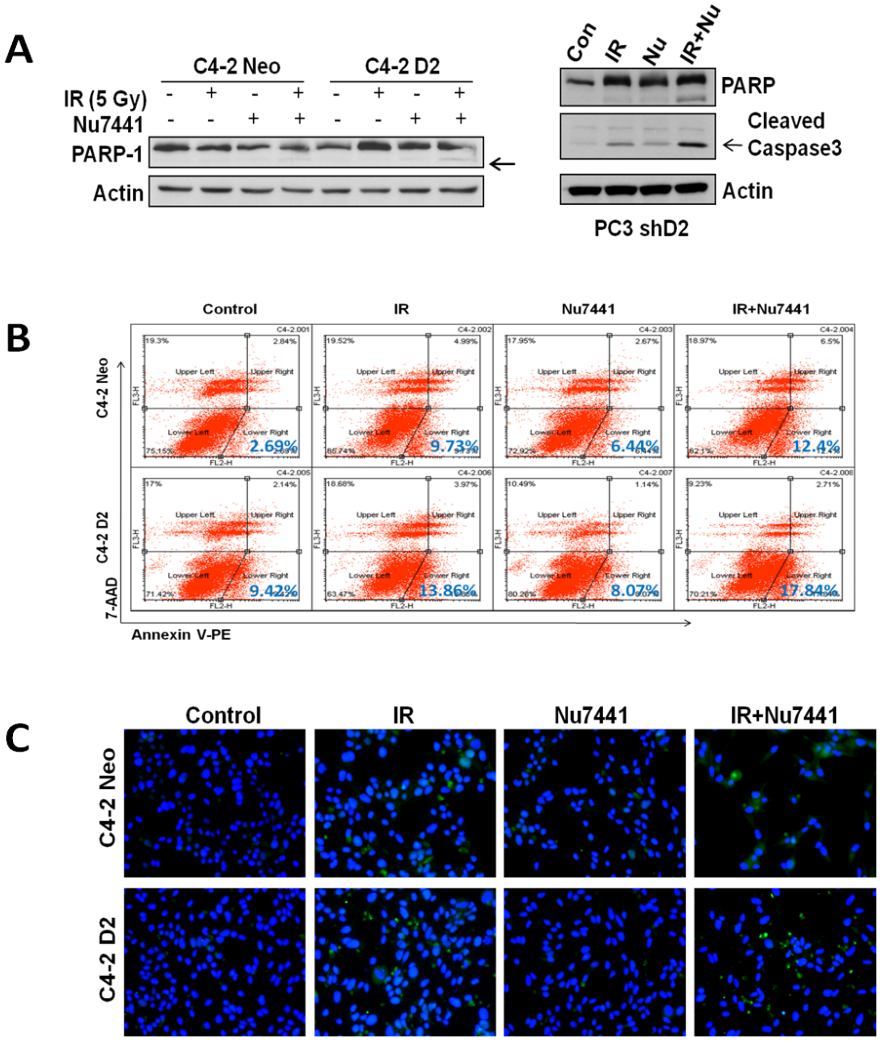

Figure 5: Apoptotic cell death was prominent in DAB2IP-proficient PCa cells. (A) Western blot analysis for detecting the PARP-1 cleavage and Caspase 3. (B). Detection of % Apoptosis in response to IR, NU7441 and IR+NU7441 by Annexin V staining. (C) TUNEL assays were performed in C4-2 Neo and C4-2 D2 cells after treatment with IR or NU7441+IR

Reportable outcomes
We will be reporting these results in the form of a manuscript for publication in a peer reviewed journal. In addition, we have observed an important event while we were investigating the mechanism of radiosensitization using NU7441. We found that DAB2IP-deficient PCa cells show dramatic induction of autophagy after treatment with radiation and NU7441. This is a novel function of DAB2IP in suppression of IR induced and DNA-PKcs associated autophagy in PCa cells.

Conclusion:
We have made significant progress which we described under Task 1 to 3. In addition, we also attached the draft of the manuscript which is in the final stage of submission to a peer reviewed journal

References: References are included in the attached manuscript

Appendices:
The results are now described in the attached manuscript by Yu et al which will be submitted to the Journal of Neoplasia

DAB2IP Regulates Prostate Cancer Cell Survival In Response to ionizing radiation and a DNA-PKcs inhibitor NU7441

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Running title:

Key words: PCa; DAB2IP; DNA-PKcs inhibitor; Radiosensitization; Autophagy

Abbreviations: PCa: Prostate Cancer; DAB2IP, DOC-2/DAB2 interactive protein; IR: Ionizing radiation; RT: Radiation Therapy; DSBs: Double strand breaks; DER: Dose Enhancement Ratio; EF: Enhancement Factor.

Conflict of interest: None

Abstract

Radiation therapy is both a common and effective strategy for the treatment of localized prostate cancer. However, a proportion of locally advanced cancers develop radiation resistance and recur after therapy, therefore the development of radiation sensitizing compounds is essential for treatment of these tumors. DAB2IP (DOC-2/DAB2 interactive protein) which is a novel member of the Ras GTPase-activating protein family and a regulator of PI3K/Akt activity, is often downregulated in aggressive prostate cancer (PCa). Our previous studies have shown that loss of DAB2IP results in radioresistance in PCa cells primarily due to faster DNA double-strand break (DSB) repair kinetics, robust G2-M check point control and evasion of apoptosis. A novel DNA-PKcs inhibitor NU7441 can significantly enhance the effect of radiation in DAB2IP-deficient PCa cells. This enhanced radiation sensitivity after NU7441 treatment is primarily due to delayed DNA double-strand break (DSB) repair. More importantly, we reported that DAB2IP-deficient PCa cells show dramatic induction of autophagy after treatment with radiation and NU7441. Immunoblotting analysis showed that the autophagy-associated proteins such as LC3B and Beclin1 deregulated in DAB2IP proficient PCa cells. We observed decreased phosphorylation of S6K and mTOR in DAB2IP-overexpressed cells. Taken together, our study clearly shows that NU7441 is a potent radiosensitizer in aggressive PCa cells. More importantly, our study indicates that DAB2IP may act as an important factor in PCa cell death after combined treatment with NU7441 and radiation.

Introduction

Prostate cancer (PCa) is the most common type of non-skin cancer and the second

leading cause of cancer related death in U.S men [1]. Radiation therapy (RT) provides excellent local control and increased overall survival when used a treatment for PCa [2]. However, a significant proportion of high-risk patients display radiation resistance and develop metastatic disease in less than 5 years [3]. Elucidation of biomarkers and their effects on mediating therapeutic resistance may allow physicians to personalize care based on genotype. DAB2IP/AIP1 (DOC-2/DAB2 interactive protein, or ASK1 interacting protein), a member of the RAS-GTPase activating protein (RAS-GAP) family, acts as a tumor suppressor but is often downregulated in aggressive PCa [4]. In addition, our previous work demonstrated that loss of DAB2IP expression results in increased radioresistance in both PCa cells and normal prostate epithelia [5, 6]. Therefore, elucidating the mechanism by which loss of DAB2IP induces radioresistance will provide useful information in identifying new strategies to sensitize DAB2IP-deficient PCa cells to RT.

DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase (DNA-PK) and member of the phosphatidylinostitol 3-kinase-like family, plays a dominant role in non-homologous end joining (NHEJ) mediated DNA double-strand break (DSB) repair [7]. Furthermore, DNA-PKcs may play a role in initiating DNA-DSB induced apoptosis [8, 9]. Upon recruitment to DSB sites, DNA-PKcs phosphorylates downstream targets involved in DNA repair response and promotes direct ligation of broken DNA ends. Accordingly, suppression of DNA-PKcs leads to ineffective DSB repair and increases the cytotoxicity of ionizing radiation and other DSBs-inducing agents [10]. Based on the important role of DNA-PKcs in NHEJ, inhibition of DNA-PKcs is, therefore, an attractive approach to modulating resistance to radiotherapy. One of the primary goals of this study is to develop strategies to overcome

DAB2IP downregulation induced radioresistance and improve the efficacy of radiotherapy in PCa using NU7441, a potent and specific inhibitor of DNA-PKcs.

Recent studies suggest that DNA-PKcs is involved in DNA damage induced autophagy. Specifically, inhibition of DNA-PKcs sensitized malignant glioma cells to radiation-induced autophagic cell death [11]. However, autophagy, which normally results in degradation of damaged or potentially dangerous proteins and organelles, may have a pro-survival function which protects cells from various forms of cellular stress [12]. Several studies indicate that pharmacologic or genetic inhibition of autophagy can enhance cancer treatments by sensitizing cancer cells to both radiation and chemotherapy [13]. Based upon these reports, we analyzed the levels of autophagy in NU7441 treated DAB2IP-deficient and proficient PCa cells to investigate whether suppression of DNA-PKcs can confer to irradiation induced autophagy in PCa cells. In this study, we show a novel function of DAB2IP in suppression of IR induced and DNA-PKcs associated autophagy in PCa cells. Although treatment with NU7441 significantly enhanced the effect of radiation in DAB2IP-deficient PCa the combination of NU7441 with overexpression of DAB2IP resulted in greater radiation sensitization due to autophagy inhibition.

Methods and Materials

Cell culture and irradiation

Prostate cancer cell lines C4-2 and PC3 were grown in T medium (Invitrogen, Carlsbad , CA) with 5% fetal bovine serum (Hyclone, Hudson, NH) at 37^{o}C with 5% CO2 in a humidified chamber. C4-2 neo (DAB2IP deficient) and C4-2 D2 (DAB2IP overexpressed) were generated

from C4-2 cells, and PC3 Con (DAB2IP positive) and PC3 KD (DAB2IP knocked down) were generated from PC3 cells as described previously [5]. All cells were irradiated in ambient air using a ^{137}Cs source (Mark 1-68 irradiator, JL Shepherd & Associated) at a dose rate of 3.47 Gy/min at room temperature.

Clonogenic survival assay

Exponentially growing cells were trypsinized and counted using a Coulter counter (Beckman Coulter, Fullerton, CA). Cells were diluted serially to appropriate concentrations and plated into 60 mm dishes in triplicate. After 3 hours of incubation, cells were treated with increasing doses of IR (2, 4, 6, 8 Gy) or NU7441 (1 μM) or NU7441 + IR. After 10-14 days, cells were fixed and stained with 4% formaldehyde, 0.05% crystal violet in phosphate buffered saline. Colonies containing >50 cells were counted. Surviving fraction was calculated as (mean colony counts)/[(cells inoculated) × (plating efficiency)], in which plating efficiency was defined as (mean colony counts)/(cells inoculated for unirradiated controls). The data are presented as the mean ± SEM of at least three independent experiments. The curve $S = e^{-(\alpha D + \beta D2)}$ was fitted to the experimental data using a least square fit algorithm using the program Sigma Plot 11.0 (Systat Software, Inc.). From these curves the dose leading to a survival of 0.1 to 0.6 was then calculated and determines the DER (dose for radiation alone divided by the dose for radiation plus drugs).

Retrospective Cohort Analysis

Patients with high-risk disease (stage T3a or greater, or Gleason score (GS) > 7, or Prostate Specific Antigen (PSA) > 20) treated with definitive RT between 2005-2011 at UT Southwestern were identified. Immunohistochemistry (IHC) analysis for DAB2IP protein was

performed on their biopsy specimens. DAB2IP status was scored in the tumors by an expert GU pathologist. Biochemical recurrence-free survival (BRFS) of patient cohorts with and without DAB2IP loss was determined using the Phoenix definition. Log rank test was used to correlate BRFS with DAB2IP levels. Univariate analysis of BRFS to pretreatment PSA, GS, stage, age, DAP2IP status was performed.

Antibodies

Anti-phospho-Histone γH2AX (Ser139) were obtained from Millipore (Billerica, MA). 53BP1, mTOR, pmTOR(S2448), pS6K(T389), LC3B and Beclin1 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent dye–conjugated secondary antibodies were obtained from Invitrogen Corporation (Carlsbad, CA).

The detection of γH2AX and 53BP1 foci

The 53BP1 and phosphorylation of H2AX (γH2AX) were used as an indicator of DNA DSB. Cells were cultured for the indicated times to repair DNA lesions after 2 Gy of irradiation either alone or combined with 2 μM NU7441. The cells were fixed in 4% paraformaldehyde/PBS for 30 min, permeabilized in 0.5% Triton X-100/PBS for 15 min, and blocked in 5% bovine serum albumin for 30 min. The samples were incubated with anti-phospho-Histone γH2AX (Ser139; 1:2,000) and 53BP1 (1:500) for 1 h, washed in PBS for 10 min three times, and incubated with Alexa Fluor 488-conjugated goat anti-rabbit and rhodamine red-conjugated goat anti-mouse secondary antibodies (1:1,000) for 1 h. The cells were washed for 10 min three times and mounted using VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The number of γH2AX and 53BP1 foci was examined

using a fluorescence microscope.

Cell Cycle Analysis

The treatment or control cells were harvested and fixed using 75% ethanol either immediately or at the indicated time after radiation and radiation+NU7441. The cells were resuspended in PBS containing 1 μg/ml RNase A (Sigma, St. Louis, MO, USA), incubated for 30 minutes at 37°C, and stained with 100 μg/ml propidium iodide (Sigma, St. Louis, MO) for 15 min at 4°C. The cell cycle distribution was analyzed using flow cytometry, and a minimum of 10,000 cells per sample were counted.

Detection of acidic vesicular organelles

Cells were grown in 60mm dishes and allowed to attach overnight. The cells were treated with radiation or radiation+NU7441 as indicated, and then were incubated with 1 μg/ml acridine orange/PBS for 15 min, washed with PBS, and examined under LSM 510 laser-scanning confocal microscope (Zeiss) at 63X magnification. Untreated cells were also cultured for 3 days as a negative control. The samples were collected for FACScan and analyzed using Flowjo 8.7.1(Tree Star, Inc.) software to quantify cells that were positive for acidic vesicular organelles.

Statistical analysis

Data are presented as the mean ± SEM of at least three independent experiments. The results were tested for significance using the unpaired Student's t test.

Results

NU7441 sensitizes DAB2IP-deficient cells to irradiation

To test the effect of NU7441 on the radiosensitivity of DAB2IP positive and negative

prostate cancer cells endogenous DAB2IP expression was knocked down using shRNA in PC3 cells (PC3 KD). In addition, we stably transfected and expressed exogenous DAB2IP protein in a DAB2IP null PCa cell line C4-2 and marked as C4-2D2, whereas, the control cell line marked as C4-2 Neo. Previously we showed the immunoblot analyses to confirm DAB2IP expression in PC3 and C4-2 cells [5].

As shown in Figure 1, *A* and *B* shRNA-mediated suppression of DAB2IP significantly increased radiation resistance in PC3 cells (Figure 1*B*). In contrast, DAB2IP overexpression resulted in a significant radiation sensitizing effect in C4-2 D2 cells (Figure 1*A*). Using colony formation assays, we found that NU7441 significantly sensitized DAB2IP-deficient (C4-2 neo and PC3 KD) cells to IR. Surviving fraction at 2 Gy (SF_2) for C4-2 neo and PC3 KD cells were reduced from 0.65 ±0.023 and 0.86 ±0.04 to 0.24 ±0.04 and 0.55 ±0.06 respectively. Here we confirm, as previously shown [5], DAB2IP expressing cells are more radiosensitive when compared to DAB2IP deficient cells. Furthermore we found that NU7441 further sensitized DAB2IP efficient cells to IR. SF2 values of C4-2 D2 and PC3 Con vector cells were decreased from 0.31 ±0.06 and 0.58 ±0.06 to 0.14 ±0.02 and 0.02 ±0.007 respectively. The radiation sensitizing effect of NU7441 is dose dependant (Figure 1*C*). These in vitro results indicate that NU7441 can radiosensitize both DAB2IP positive and negative PCa cells to radiation.

NU7441 decreases DNA DSB repair in DAB2IP positive and negative PCa cells

The extent of DNA DSBs generated and the ability of tumor cells to repair the damage largely determine the efficacy radiotherapy. Therefore, to investigate whether the radio-sensitizing effect of NU7441 on DAB2IP-deficient cells was a result of increased

compromised DSBs repair, cells were subjected to immunofluorescence staining for phosphorylated histone H2AX (γH2AX) (green) and 53BP1 (red) (Figure 2, *A* and *B*), DNA DSBs repair kinetics were determined by counting the separate foci at each time point. In this study, C4-2 neo and C4-2 D2 cells were exposed to 2 μM Nu7441 for 1 h before 2 Gy IR treatment and the cells were collected at various times as indicated. As shown in Figure 2*C*, rapid induction of DNA damage was detected within 30 min in all of the cells. In untreated cells, DNA DSBs foci were almost abolished at 24 h after IR whereas, a significant number of DSBs remained at 24 hours in NU7441 treated cells; similar results were present in PC3 KD cells (Figure 2, *B* and *D*). The residual number of DSB foci per nucleus in DAB2IP overexpressed C4-2 cells was significantly higher than the control cells 30 minutes postirradiation, however, detectable foci fell to the same level as control cells 24 hours post irradiation. This data indicates that DAB2IP overexpression in C4-2 cells promotes IR induced DNA DSBs but has no effect on repair kinetics. Taken together, these results show that NU7441 can lead to defective DNA damage repair in response to IR even in DAB2IP-deficient metastatic PCa cells.

In mammalian cells, IR-induced cell cycle arrest is necessary to maintain genomic stability and is correlated to cell survival. In this study, the effect of NU7441 on cell cycle distribution was investigated by flow cytometry. As shown in Figure 3, *A*-*D*, treatment with 2 μM NU7441 resulted in robust G2/M arrest 24 hours after irradiation (3 Gy) in both DAB2IP-deficient or overexpressed cell lines. We further compared C4-2 neo and C4-2 D2 cells and showed the percentage of G0/G1 phase cells in C4-2 D2 was more than that in neo (control) line. The cell cycle results indicate that DAB2IP alone causes significant G0/G 1

arrest in C4-2 cells and is augmented after treatment with either IR or NU7441. Whereas DAB2IP deficient cells showed moderate increase in G0/G 1 arrest after NU7441 treatment. However, both cell lines showed robust G2/M arrest at 24 h in response to combined treatment.

DAB2IP overexpression impairs irradiation-induced autophagy

Autophagy is the mechanism of proteolysis has been involved in both cell survival and cell death [14]. In general, autophagy protects cells from stressful conditions such as nutrient deprivation. There are reports also indicating that autophagy protects cells from radiation [15]. To test whether DAB2IP is involved in PCa cell autophagy, we analyzed the formation of acidic vesicular organelles (AVO) using acridine orange (AO) staining with fluorescence imaging and flow cytometry. During autophagy AO accumulates in acidic compartments such as autolysosomes. Using a laser scanning confocal microscope, we found that the autophagic signal increases in DAB2IP deficient C4-2 neo cells after IR, NU7441 and the combination of NU7441 and IR treatment. However, across the all three treatment arms, there was a significant decrease in the amount of autophagy in the DAB2IP overexpressing cell line C4-2 D2 (Figure 4*A*). To further confirm this result, we performed flow cytometric analyses of AO-stained cells. As shown in Figure 4*B*, DAB2IP deficient cells display significantly more autophagy than DAB2IP expressing cells. We also performed the immunostaining with LC3B antibody (Figure 4*C*) and the results were consistent with Figure 4, *A* and *B*. We further subjected C4-2 neo cells to IR combined with an autophagy inhibitor, Baf A1. The clonogenic assay clearly showed that Baf A1 treatment enhanced radiation induced cell death in DAB2IP deficient PCa cells (Figure 4*D*). Based on these results, we propose that the decreased

resistance to IR in DAB2IP overexpressed C4-2 cells may be partially due to inhibition of autophagy.

DAB2IP overexpression suppresses the mTOR-S6K pathway

To further explore the role of DAB2IP in autophagy, we determined the expression levels of the microtubule-associated protein LC3B. LC3B exists in a cytosolic form, LC3B-I, and a LC3B-II form that is conjugated to phosphatidylethanolamine [16, 17]. Increased LC3B-II levels are closely associated with the number of autophagosomes and serve as a good indicator of autophagosome formation [18]. As shown in Figure 5, *A* and *B*, overexpression of DAB2IP impairs both IR and NU7441 mediated induction of LC3B-II in C4-2 cells. The expression of Beclin1, which participates in autophagosme formation, was further determined by western blot and noticed that Beclin1 expressed lower levels in DAB2IP overexpressed C4-2 cells.

It is reported that Akt-mTOR pathway negatively regulates autophagy [19]. To investigate how this signaling cascade is operated in DAB2IP associated autophagy inhibition in C4-2 cells, phosphorylation of mTOR was measured in both cell lines (Figure 5*B*). Compared with C4-2 neo cells there was a dramatic inhibition of phosphorylated mTOR in C4-2 D2 cells. Furthermore, studies have shown that S6K is a critical downstream effector of the mTOR signaling pathway [20]. We observed decreased phosphorylation of S6K in DAB2IP overexpressed C4-2 D2 cells (Figure 5*B*). Although mTOR-S6K activation is known to suppress autophagy in mammalian cells, emerging studies have indicated that, in certain situations, the mTOR-S6K pathway positively regulates autophagy [21-23]. Together, our findings show that DAB2IP may suppress IR and NU7441 induced autophagy through the

mTOR-S6K pathway.

Loss of DAB2IP Correlates with Decreased Biochemical Recurrence Free Survival

To determine whether loss of DAB2IP in PCa results in clinically relevant radiation resistance we performed a retrospective cohort study of high risk prostate cancer patients treated with definitive RT (Figure 6). Patient's DAB2IP status was determined using IHC and patients were followed for biochemical recurrence as mentioned in methods. Twenty four patients treated for high risk prostate cancer were evaluated. DAB2IP loss was seen in 8 patients (33.3%) whereas 16 patients (66.7%) expressed DAB2IP. Median follow up for DAB2IP patients was 19.4 months (m) (range 8.2 to 57.8 m) and 23.9 m (range 8.8 to 74.0 m) for the DAB2IP deficient group. Patients expressing DAB2IP exhibited markedly improved BRFS compared to patients with loss of DAB2IP (p=0.027; log-rank test). The estimated 2 yr BRFS was 90% and 68.5% respectively for the DAB2IP present and deficient groups. At 4 yrs BRFS was 90% and 34.3% for the present and deficient groups respectively. Univariate analysis demonstrated DAB2IP status as the only variable with significant association to BRFS at this point of follow up.

Discussion

DAB2IP/ASK-interacting protein 1, a potential tumor suppressor gene, is often downregulated in PCa primarily due to altered epigenetic regulation of its promoter [24]. It functions as a key scaffold protein to modulate cell proliferation, survival, and apoptosis by coordinating PI3K, Akt, and the ASK1 pathway [25, 26]. Our previous work indicated that loss of DAB2IP expression in PCa cells greatly increases radiation resistance in vitro [5, 6]. To

determine whether our previous in vitro findings were clinically relevant, we performed a retrospective cohort study and determined that loss of DAB2IP lead to significantly increased rates of biochemical failure after radiation therapy (Figure 6).

DNA-PKcs, a key component of the non-homologous end-joining (NHEJ) pathway, plays a dominant role in DNA double-strand break repair, genomic integrity, maintaining telomere stability [7, 27]and upregulated in various cancers [28, 29]. It is also reported that increased DNA-PKcs expression and kinase activity are closely associated with radio- or chemo-resistance [30, 31]. Inhibitors of DNA-PKcs such as NU7441have been developed to enhance radiotherapy-based local tumors control [32]. In this study, we clearly show that adjuvant treatment with NU7441 can overcome PCa radioresistance caused by loss of DAB2IP (Figure 1). NU7441 mediated radiosensitization in PCa cells is mainly contributed to delayed IR induced DSB repair.

A recent study in glioma-initiating cells demonstrated that DNA-PKcs is involved in autophagy in response to irradiation. In these cells, suppression of DNA-PKcs sensitizes cells to IR-induced autophagic cell death [11]. Autophagy often serves as a cell survival mechanism in response to various stresses, including IR treatment [15, 33]. Whether the inhibition of DNA-PKcs can induce autophagy in PCa cells and the precise role of this catabolic process is not clear. In this study, AVOs staining was used to compare cellular autophagy response to IR with or without NU7441 treatment. We noticed that NU7441 treatment can promote both IR induced and basal level of cell autophagy. The autophagy marker protein LC3B is induced upon treatment with NU7441 and Bafliomycin A1 an autophagy inhibitor, significantly enhanced radiation sensitivity (Figure 4 and 5).. Moreover, these results demonstrate that the

involvement of DNA-PKcs in autophagic response is a general phenomenon and not restricted to radiation only.

Our results found that DAB2IP attenuated IR and NU7441 induced autophagy. Furthermore, we observed that LC2B and Beclin1 are downregulated in cells that overexpress DAB2IP. These results suggest that DAB2IP mediated radio-sensitization of PCa cells partially through inhibition of autophagy. The mTOR-S6K pathway is postulated to be a negative regulator of mammalian autophagy[19]. In contrast, we show that mTOR-S6K pathway was inactivated in DAB2IP overexpressed PCa cells. Studies show that mTOR can also positively modulate autophagy. Klionsky et al reported that mTOR inhibition by rapamycin or siRNA-mediated silencing of S6K expression reduced 6-TG-induced autophagy in human colorectal cancer cells [21]. Scott et al suggested that S6K is essential for autophagy in fat cells of *Drosophila* [22]. Consistent with this report, our results showed that the phosphorylation of S6K almost totally attenuated in DAB2IP overexpressed PCa cells. Notably, DAB2IP functions as a scaffold protein to inhibit the PI3K-Akt pathway via a direct protein interaction with PI3K through its PR domain [25].

Autophagy-defective cells also show an increase in DNA-DSB in response to various stresses, including IR [13, 34]. To further evaluate the role of autophagy in DNA-DSB repair we found that DAB2IP proficient PCa cells exhibit higher level of DNA damage compared to control cells after IR treatment. We believe that the loss autophagy in DAB2IP proficient cells is the major contributing factor toward the impaired DNA damage. Moreover, recent work indicates that autophagy can promote tumor cell survival and inhibition of autophagy leads to significant tumor regression [35]. It is conceivable that DAB2IP induced autophagy impairment

contributes to the suppression of tumor growth and aggressiveness.

In summary, the results of this study demonstrate that inhibition of DNA-PKcs enhances the effect of IR DAB2IP deficient radioresistant human PCa cells. Moreover, our findings demonstrate a role for DAB2IP in inhibiting mTOR-S6K pathway and suppressing autophagy. Based on these results, DAB2IP appears to be an excellent radio-therapeutic target in treatment of prostate cancer.

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Figure Legends

Figure 1. DNA-PKcs inhibitor NU7441 increased the radiosensitivity in both DAB2IP deficiency and efficiency prostate cancer cell line, whereas the overexpression of DAB2IP further sensitized prostate cancer cells to IR. (A) The survival curves of DAB2IP-overexpressed C4-2 D2 cells and control C4-2 neo cells after combination treatment with 1 μM NU7441 and irradiation at doses of 0-8 Gy. The data are expressed as the means ± standard deviations from three independent experiments. (B) The survival curves of

DAB2IP-surrpressed PC3 KD cells and control PC3 Con cells after combination treatment with 1 μM NU7441 and irradiation at doses of 0-8 Gy. The data are expressed as the means ± standard deviations from three independent experiments. (C) The survival curves of PC3 KD cells after combination treatment with 0.5-5 μM NU7441 and irradiation at doses of 0-8 Gy. The data are expressed as the means ± standard deviations from three independent experiments.

Figure 2. Decreased capacity of DNA double-strand break repair in NU7441 treated prostate cancer cell lines. (A) C4-2 D2 and C4-2 Con cells were treated with 2μM NU7441, 30 min later were irradiated with 2 Gy γ-ray and immunostained for 53BP1 (red) and phospho-γH2AX (green) foci at the indicated time points after irradiation. 53BP1 and phospho-γH2AX focis were counted for each time point (average, 50 nuclei). (B) PC3 KD cells were treated with 2μM NU7441, 30 min later were irradiated with 2 Gy γ-ray and immunostained for 53BP1 (red) and phospho-γH2AX (yellow) foci at the indicated time points after irradiation. 53BP1 and γH2AX foci were counted separated for each time point (average, 50 nuclei). (C) and (D) DNA repair kinetics between these cells was obtained by plotting the number of remaining foci against time.

Figure 3. Treatment with 2μM NU7441 results in a robust G2/M cell arrest 24h after 3Gy irradiation in either C4-2 Neo or C4-2 D2 cells. (A) C4-2 D2 and C4-2 neo cells were treated with 2μM NU7441 or combination treated with 3 Gy and collected 0, 2, 6, and 24 h post-treatment. PI staining was applied to detect the distribution of cell cycle response to

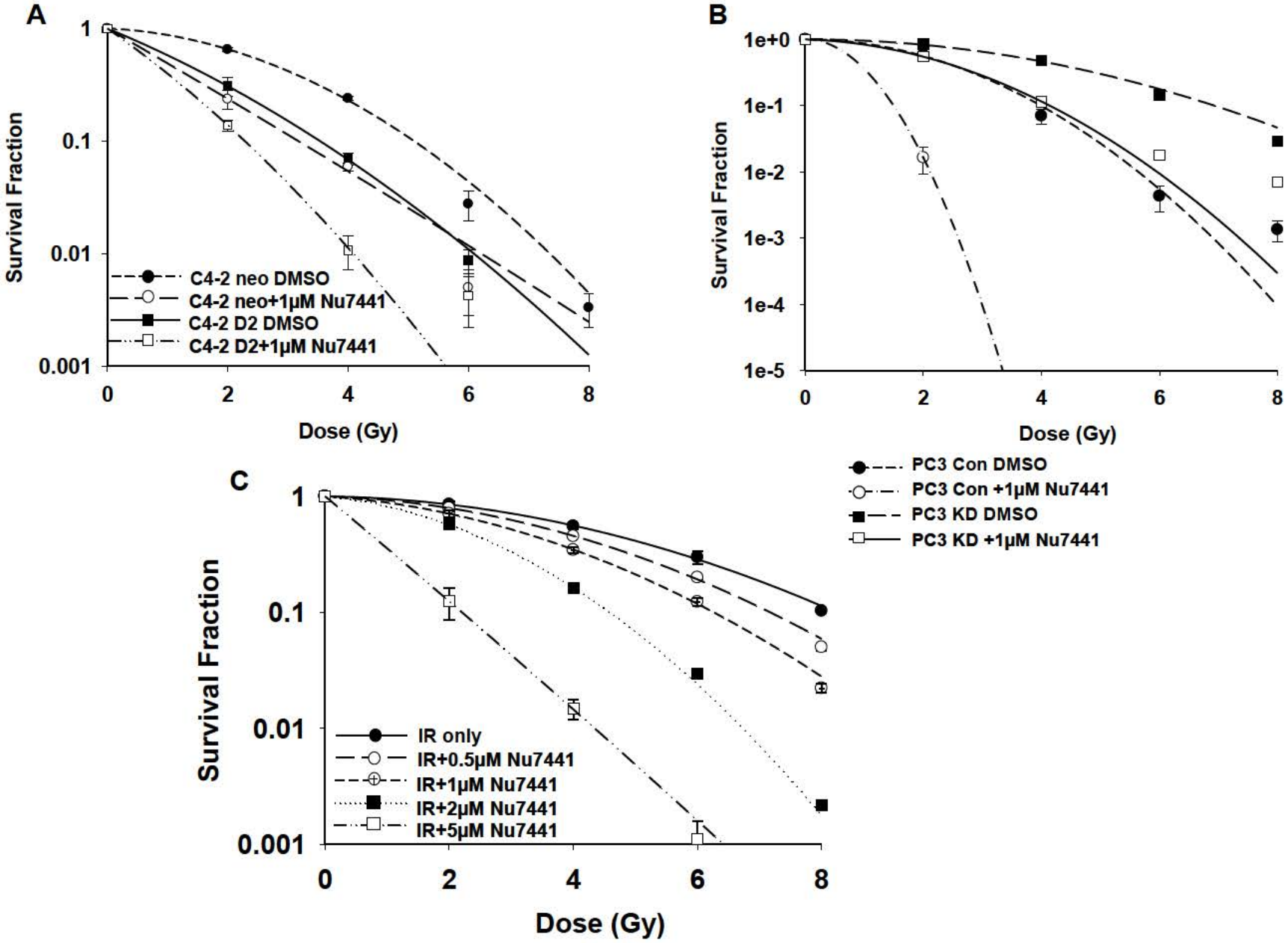
ionizing irradiation by flow cytometry. (B) Cell cycle distribution of cells 24h after NU7441 treatment and (or) 3 Gy irradiation.

Figure 4. DAB2IP overexpression suppressed IR- or NU7441+IR-induced autophagy in C4-2 cells. (A) The cells were grown in the presence or absence of NU7441 treatment and 5 Gy irradiation for 72 hrs and the fluorescence images of acridine orange-stained AVO-positive cells are shown. (B) Flow cytometry analysis to assess autophagy. The treatment was performed as described in A. (C) Immunostainng analysis using LC3B antibody. The treatment was performed as described in A.

Figure 5. DAB2IP overexpression inactivated mTOR-S6K pathway and suppressed the expression of some autophagy associated proteins. (A) The phosphorylation mTOR, S6K and the expression of autophagy associated Beclin 1 and LC3B was determined by western blot analysis 24 h after 5-Gy irradiation. (B) The phosphorylation mTOR, S6K and the expression of autophagy associated Beclin 1 and LC3B was determined by western blot analysis at the indicated time points.

Figure 6. IHC staining of DAB2IP in high risk prostate cancer patients. **(**A) Representative patients with positive DAB2IP expression. (B) Representative patient with DAB2IP loss. (C) Biochemical recurrence free survival plotted by patient's DAB2IP status.

Figure 1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Survival Fraction
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0.1
0.01
0.001
0
2
4
6
8
Dose (Gy)
C4-2 neo DMSO
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C4-2 D2 DMSO
C4-2 D2+1μM Nu7441
B
Survival Fraction
1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
0
2
4
6
8
Dose (Gy)
PC3 Con DMSO
PC3 Con +1μM Nu7441
PC3 KD DMSO
PC3 KD +1μM Nu7441
C
Survival Fraction
1
0.1
0.01
0.001
0
2
4
6
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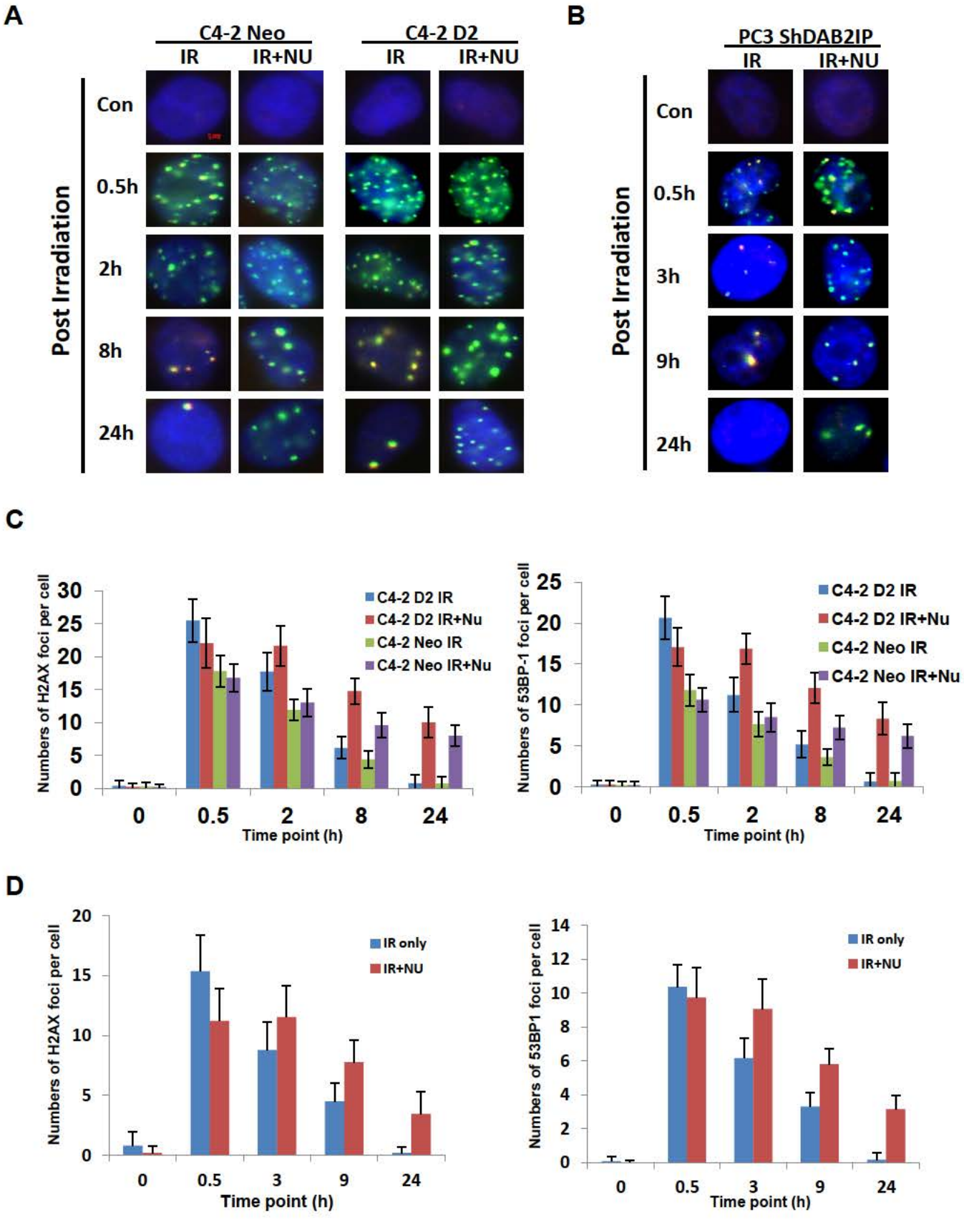
Figure 2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IR+NU
IR
IR+NU
Post Irradiation
Con
0.5h
2h
8h
24h
B
PC3 ShDAB2IP
IR
IR+NU
Post Irradiation
Con
0.5h
3h
9h
24h
C
Numbers of H2AX foci per cell
C4-2 D2 IR
C4-2 D2 IR+Nu
C4-2 Neo IR
C4-2 Neo IR+Nu
Time point (h)
Numbers of 53BP-1 foci per cell
C4-2 D2 IR
C4-2 D2 IR+Nu
C4-2 Neo IR
C4-2 Neo IR+Nu
Time point (h)
D
Numbers of H2AX foci per cell
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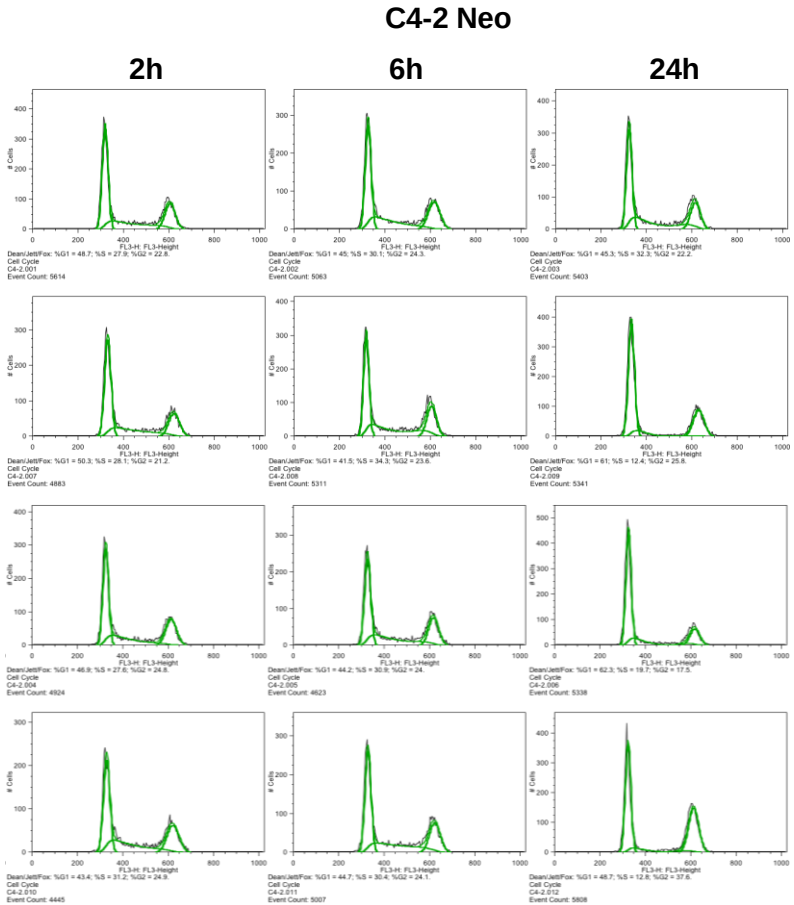
Time point (h)

Figure 3

Lan et al

A



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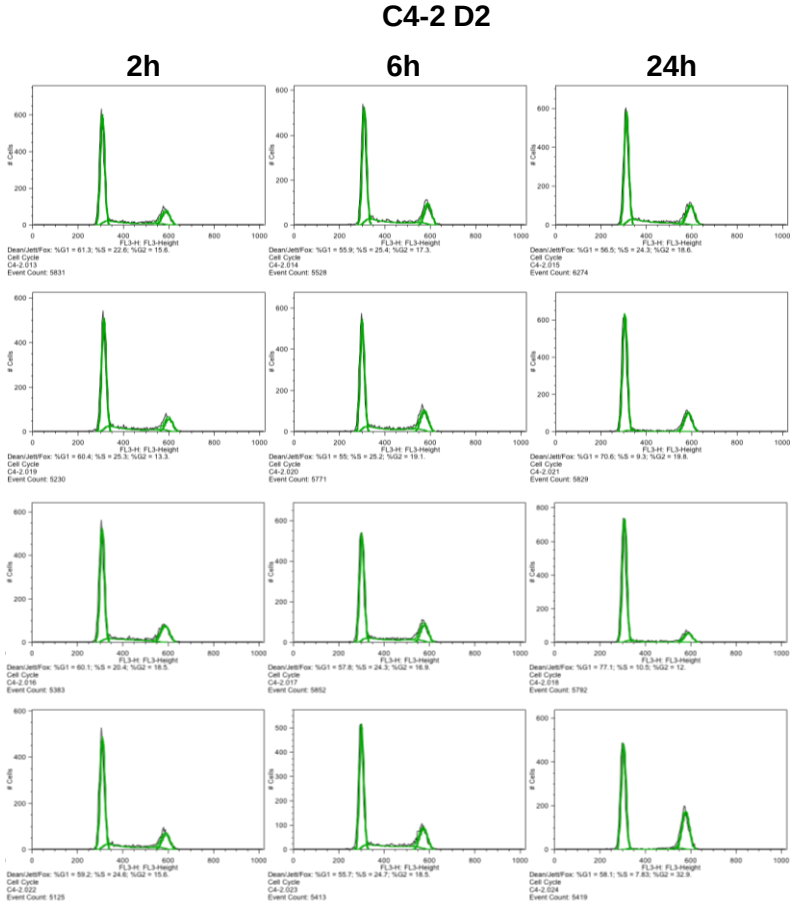

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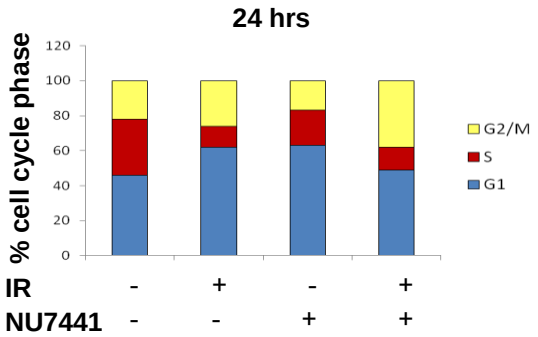

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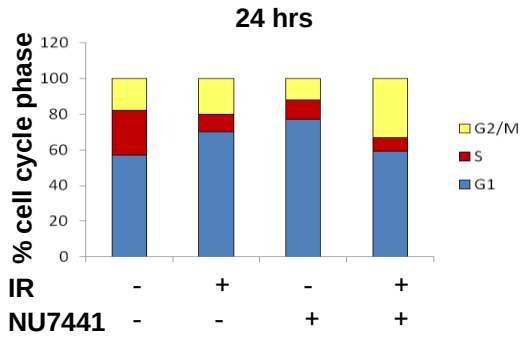
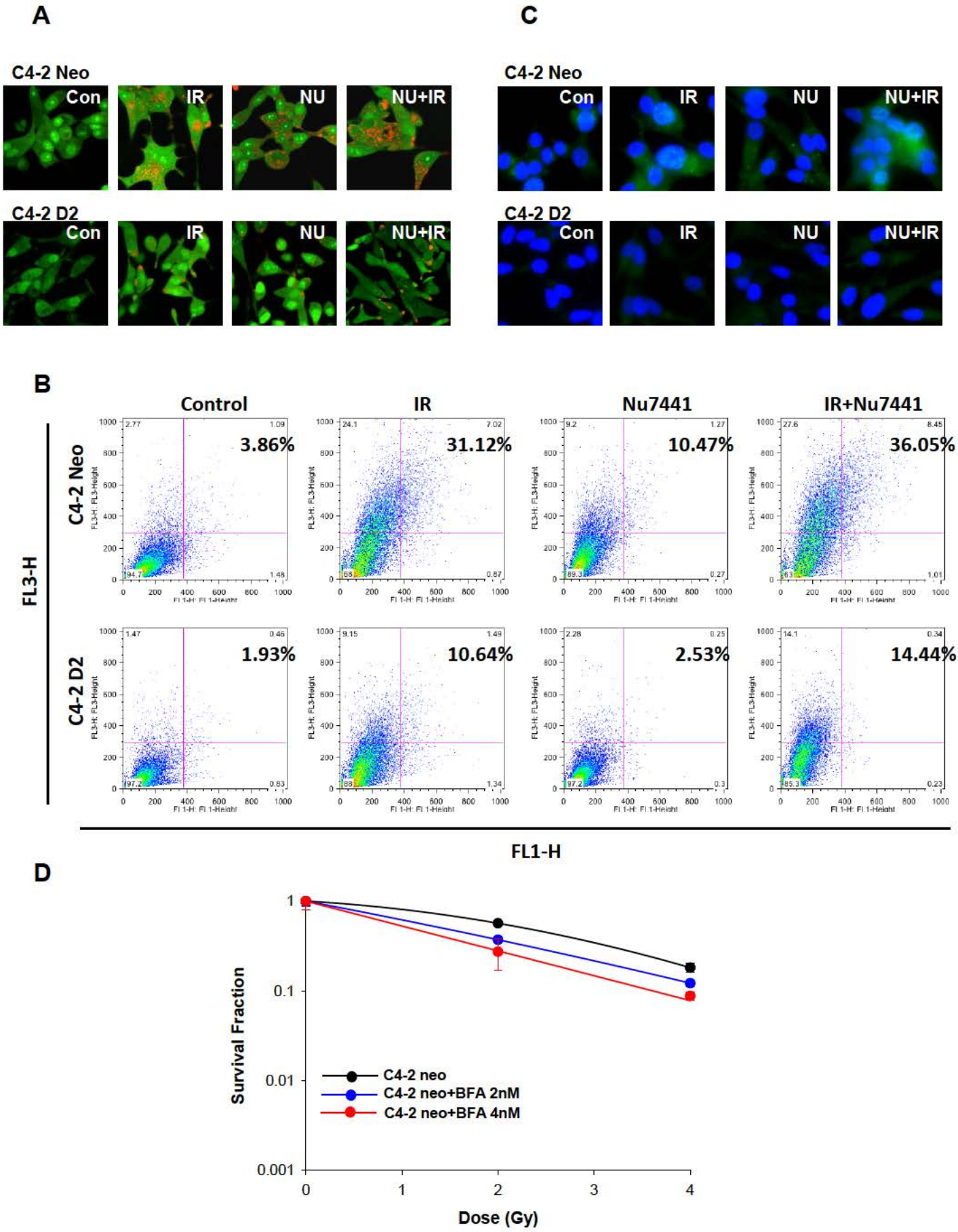


Figure 4


A
C4-2 Neo
Con
IR
NU
NU+IR
C4-2 D2
Con
IR
NU
NU+IR
C
C4-2 Neo
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IR
NU
NU+IR
C4-2 D2
Con
IR
NU
NU+IR
B
Control
IR
Nu7441
IR+Nu7441
C4-2 Neo
3.86%
31.12%
10.47%
36.05%
C4-2 D2
1.93%
10.64%
2.53%
14.44%
FL3-H
FL1-H
D
Survival Fraction
1
0.1
0.01
0.001
0
1
2
3
4
Dose (Gy)
C4-2 neo
C4-2 neo+BFA 2nM
C4-2 neo+BFA 4nM

Figure 5

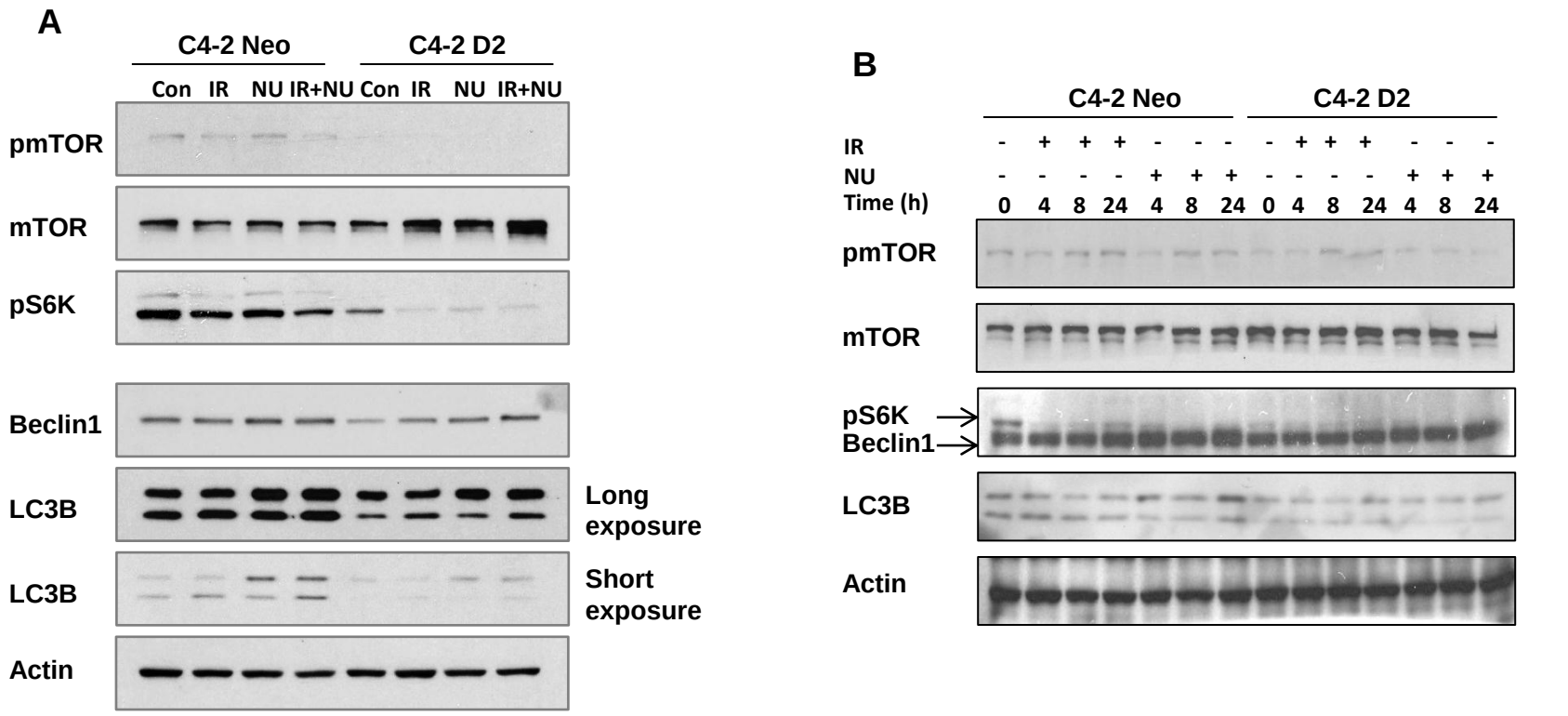
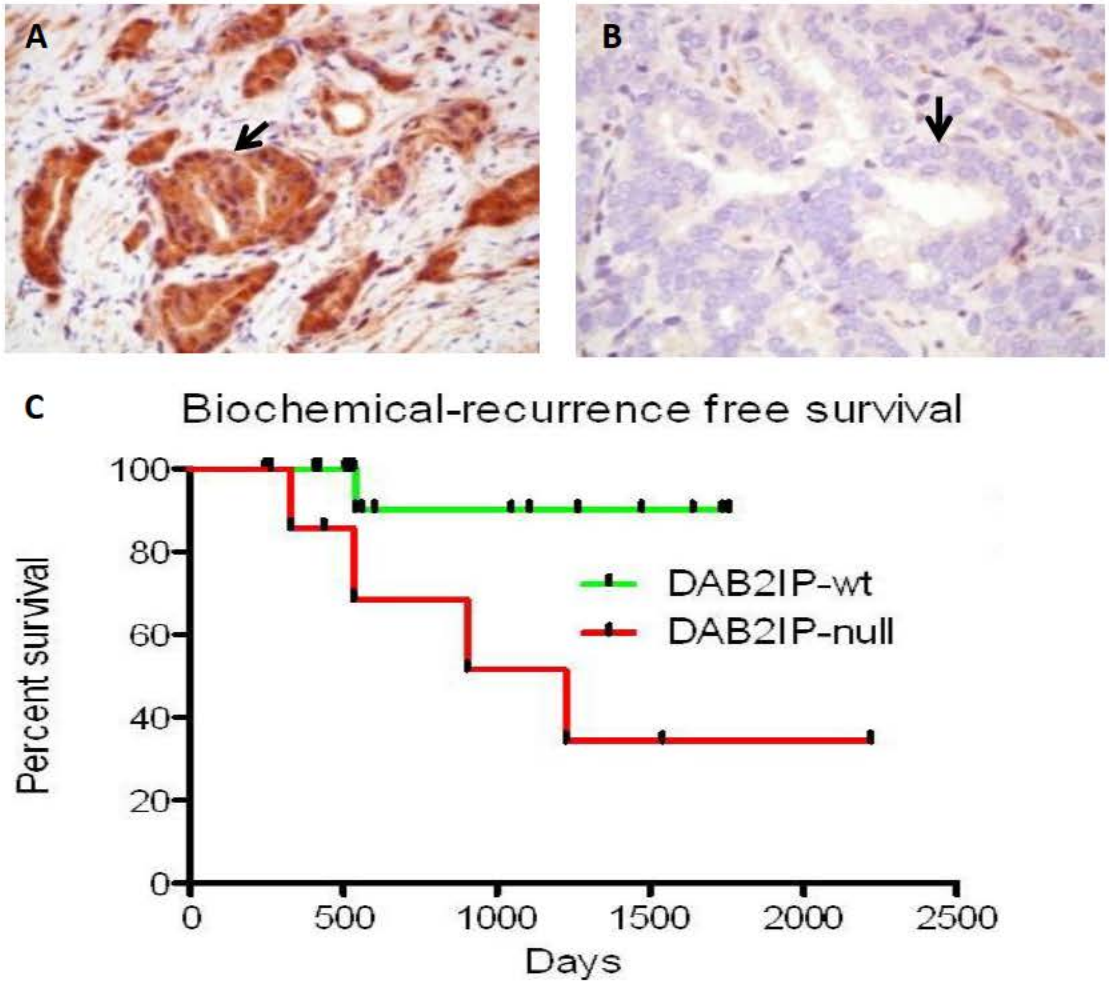

A
C4-2 Neo
C4-2 D2
Con IR NU IR+NU Con IR NU IR+NU
pmTOR
mTOR
pS6K
Beclin1
LC3B
Long exposure
LC3B
Short exposure
Actin
B
C4-2 Neo
C4-2 D2
IR
NU
Time (h)
0 4 8 24 4 8 24 0 4 8 24 4 8 24
pmTOR
mTOR
pS6K
Beclin1
LC3B
Actin

Figure 6


A
B
C
Biochemical-recurrence free survival
Percent survival
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Days
DAB2IP-wt
DAB2IP-null